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FLOW CYTOMETRY - A REVIEW OF THE TECHNIQUE WITH APPLICATION TO PHYTOPLANKTON AND BACTERIA

by

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ABSTRACT

A review of the techniques used to count phytoplankton and bacteria with particular emphasis on flow cytometry is given. It is concluded that flow cytometry is an attractive supplement to traditional microscopy but that the cost of commercial systems is too high for most laboratories. A home built system is proposed and some of the system components are enumerated.

RESUME

On passe en revue les techniques utilisées pour la numération du phytoplancton et des bactéries, en insistant surtout sur la cytométrie des cours d'eau. On conclut que cette technique vient bien compléter la microscopie traditionelle, quoique le coût des systèmes commerciaux soit trop élevé pour la plupart des laboratoires. On propose un système de fabrication interne et on en signale certains éléments.

MANAGEMENT PERSPECTIVE

This report was prepared as a response to a 1983 IWD Pacific and Yukon Region request to investigate automated methods for counting algae and bacteria.

Phytoplankton biomass is used widely as a base of reference in primary production studies, as a measure of food available for zooplankton, and as an index of lake fertility in general. While information of total biomass is relatively easily obtained through filtration techniques and direct weighing, information on species composition and size data are needed to determine growth rates and biological pathways. The classical method of obtaining this data by looking through a microscope and counting the cells or particles is labour intensive, tedious, and prone to errors from operator to operator.

This report is a review of automated cell counting techniques and equipment which have evolved, mainly in the medical field. The application of this technology to supplement traditional microscopy technique has a high potential for increasing the productivity of water quality and biological research laboratories especially if a relatively low cost system could be developed as suggested in this report.

T. Milne Dick
Chief
Hydraulics Division

PERSPECTIVE-GESTION

On a préparé ce rapport à la demande que formulaient en 1983 les régions du Pacifique et du Yukon de la DGEI afin d'explorer les méthodes automatisées de numération des algues et des bactéries.

La biomasse phytoplanctonique sert fréquemment de base de référence aux études de production primaire, comme mesure de la qualité des éléments nutritifs disponibles pour le zooplancton et comme indice de la fertilité des lacs en général. Même si l'on peut obtenir relativement sans peine de l'information sur la biomasse totale grâce aux techniques de filtration et de pesée directe, il faut tout de même obtenir des données sur la variété et la taille des espèces si l'on veut déterminer le rythme de croissance et les mécanismes biologiques. On recueille ces données à l'aide d'un microscope qui sert à compter les cellules ou les particules. Cette méthode classique demande beaucoup de main d'oeuvre, en plus d'être ennuyeuse et sujette à erreurs d'un opérateur à l'autre.

Le présent document passe en revue les techniques automatisées de numération cellulaire et du matériel qui ont évolué, surtout dans le domaine médical. En ayant recours à ces techniques pour compléter la technique traditionnelle à l'aide d'un microscope, les laboratoires pourraient atteindre un haut niveau de productivité en matière de recherche biologique et de qualité de l'eau, si un système relativement peu coûteux pouvait être mis au point, comme on le laisse entendre dans le rapport.

Le Chef,
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1.0 INTRODUCTION

This report was prepared as a result of the IWD Pacific and Yukon Region statement of "Problems and Research Needs", 1982, item 5f, requesting investigation into "automated methods for counting algae and bacteria".

Water resource managers have a need to reduce the cost of obtaining biological data either through the use of less skilled and therefore less expensive personnel or through the improved productivity of a well-trained biologist. Researchers on the other hand have a requirement to increase sample density with a view to a more accurate assessment of biomass and biocontamination. While the concept of an "automated" or "robot" biologist is somewhat premature, advances in cytometry over the last decade or so may indeed speed the measurment of phytoplankton and bacteria.

The first question is why measure phytoplankton at all? As Hallegraeff points out (Hallegraef, 1977), "phytoplankton 'biomass' (i.e., the total weight of all living phytoplankton present in a unit area at a given time) has widely been used as a base of reference in primary production studies, as a measure of food available for zooplankton, and as an index of lake fertility in general". Recently it has been discovered that a symbiosis exists between larger particles (particles of say between 10 and 100 µm in size) and particles of less than 10 µm in size (R. Daley, NWRI Pacific and Yukon Region, personal communication, 1984). Thus a requirement exists to measure particles in the size spectrum of 1 to 100 µm in order to obtain an assessment of biomass. As Hallegraef (Hallegraef, 1977) also points out, different analytical techniques give different information. Seston dry weight is relatively easy to obtain by filtration and is a measure of the energy content of the biomass but is contaminated by the presence of non-algal material. Carbon content using the C_{14} technique is also used but cannot by itself distinguish between live and dead cells. More strictly algological characteristics are chlorophyll-a measurement and cell

volume. However, chlorophyll content varies with species, light level and nutrients for example. Techniques which might assist in interpretation of results are needed. Information of not only total biomass but species composition and size data are needed to determine growth rates and biological pathways.

Supplemental microscopic analysis is required and probably will always be required in order to properly interpret results. The classical method of determining species composition and size information is to look through a microscope with a grid reticle and pressing button counters. Such a process is tedious and time-consuming. Rarely can more than a few hundred particles be examined in a sitting. As a consequence, errors from sample to sample and operator to operator may result (Kutkun, 1985, Schrag and Corn, 1970).

Three "automatic" methods or potential methods of counting particles are in use. The first methods is a Coulter counter (Coulter, 1971), the second uses some method of pattern recognition, the third is called flow cytometry. The relative advantages and disadvantages of each are discussed. The third method which appears to be the method of choice is discussed in detail. Particular attention is paid to the application of these methods to algae and bacteria.

2.0 LITERATURE REVIEW

A computerized literature search of the following data bases was done: BIOSIS Previews, CA Search, IRL Life Science, and ISMEC. The data bases were queried under the headings "CYTOMETER, CELL AND COUNTER OR SENSOR OR METER, CELL AND COUNTING AND APPARATUS OR SENSOR OR SYSTEM OR METER, PARTICLE AND COUNTER, BACTERIA AND COUNTER". It was evident that the photocopied literature would measure kilograms in mass. Selected references were examined. Because of the cost of such a search (several hundred dollars), the printed out reference list has been bound as NWRI Contribution 85-18 (ES-567), Cytometer Bibliography, supplement to this report. Copies are available. Most of the references by far

were from the medical literature. Only those references which dealt with systems as opposed to applications were examined in any detail.

3.0 ALTERNATIVE MEASUREMENT TECHNIQUES

As discussed in the introduction, three presently used or potential automatic methods of cell analysis were examined. The first method was the Coulter counter. This method, widely used for decades, is based on the change in conductivity due to a particle traversing an orifice (Coulter, 1971). An electrode is placed on either side of the orifice with a potential difference between them. As a dielectric particle traverses the aperture a voltage pulse proportional to the particle volume is produced. A pulse height analyser is then used to obtain particle size and volume distribution.

The technique apparently grew out of a requirement in rheology, the science of lubrication, to measure suspended particles in oil. The application of Coulter counters to phytoplankton measurement was discussed by Sheldon and Parsons, 1967; Battarbee and Kneen, 1982; Haffner and Evans, 1974; and by Evans and McGill, 1970. Coulter counter measurements are relatively rapid and provide particle size data for diameters between approximately 3 to 100 μm. Three problems exist with Coulter counter measurements. One is a practical one, namely clogging of the orifices occurs with regularity. This may be controlled through aperture selection and sonification of the sample prior to measurement.

The second problem is that inorganic particles are measured as well as organic particles. The third problem is that there is no species differentiation. While Coulter counter technology has proven to be a useful tool, these problems remain.

The second technology used for cell counting and differentiation is based on digital image processing.

There are a number of manufacturers of equipment specifically related to cell measurement or at least particle measurement. Several of these are:

1. Bausch & Lomb "Omnicon"
   Bausch & Lomb
   820 Linden Avenue
   Rochester, NY, 14625
2. Quantimet "Automatic Petridish Analyser"
   Imanco
   40 Pitt Drive
   Monsey, NY, 10952
3. "Magiscan Image Analysis System"
   Joyce-Loebl
   Marquisway
   Team Valley
   Gateshead NE11 OQW, England
4. "Polyprocessor C-1285"
   Hammamatsu Systems
   332 Second Avenue
   Waltham, MA, 02154
5. Leitz-Tas
   Wild Leitz
   513 McNicoll
   Toronto, Ontario

A number of custom built systems have been made (Kaman et al. 1984; Stark et al., 1984) and evaluations have been done (Goss et al., 1974). These systems have not been as extensively used as once hoped. The reasons are partially expense, but principally operational. For instance, much setup time is required and there may be difficulties in automatic focussing (Herman et. al., 1983). A more serious problem is the difficulty in distinguishing artifacts, debris particles and groups of overlapping nuclei (Stark et al., 1984). While it is easy for the eye to distinguish touching cells, to date, it has been difficult to develop reliable algorithms to accomplish the task automatically with affordable equipment.

The third technique is called flow cytometry. This combines single particle counting with the measurement of additional parameters such as fluorescence, polarization, or differential light scattering for each particle or cell. This collection of additional data is the principle advantage of cytometry over Coulter counter techniques.

The advantages of the flow cytometry technique are that it is relatively fast, compared to microscopy alone; cell sorting can be done using a number of selection criteria; cells remain alive during the counting and sorting process.

The principle disadvantage of the technique is that cell selection criteria must be limited to the relatively few parameters measured. Best results are obtained when cells under analysis are of the same type. An additional disadvantage is that commercial cytometer systems are expensive.

The flow cytometric technique is described in more complete detail in the following sections.

4.0 DESCRIPTION OF FLOW CYTOMETRY

Flow cytometry in the last fifteen years has been the subject of hundreds, if not thousands, of scientific papers (see Supplement), at least four conferences ("Flow Cytometry IV" was held in Bergen, Norway, 1980), the subject of a multi-authored book (Melamed, Mullaney and Mendelsohn, 1979), several review articles (Steinkamp, 1984, Mendelsohn, 1980; Shapiro, 1983; Miller et al., 1981; Melamed et al., 1977), and an article in a popular magazine (Herzenberg et al., 1976). However, the technique is essentially unknown and unused in the marine biological community. By far the largest users of the technique to date have been in the medical research field, specifically in immunology, hematology and oncology.

The technique is based on providing a flow cell which arranges particles to pass the sensing volume one particle at a time. Fig. 1 illustrates the principle. As a particle passes the sensing volume it is illuminated by laser or other radiation transverse to the flow

direction. The transmitted or forward scattered light is sensed. Additionally fluoresced or 90° scattered light is examined. The particle can be made to pass multiple sensing volumes.

Flow rates are typically 1000 cells/s.

In some systems, after sensing, the processed signal amplitude may be compared with a pre-set range to activate a cell sorting mechanism.

For analysis, the processed signals are then collected by a computer, stored, and displayed.

The strength of the method is that the measurements are done on each individual particle. The weakness of the method is that only a few kinds of measurement may be done. The primary detection method for cell analysis involves the use of fluorescent stains.

The cell detection criteria effectivley depend on the degree of specificity of the fluorescent tags. Steinkamp, 1984 and Shapiro, 1981, have reviewed approximately 25 cytochemical stains. Application of these staining techniques with reference to algae and bacteria are discussed in Section 11.

5.0 FLOW CELL TYPES

Essentially all of the flow cells used in flow cytometry fall into two categories namely; those based on ultrasonic droplet formation and sensing each droplet, and those based on the sensing of particles in a closed cell before any droplets are formed. Shapiro et al., 1983, compared the relative merits of several cells.

Both types of cells are based on the principle of hydrodynamic focussing illustrated in Fig. 2 (Crossland-Taylor, 1953). A good explanation for the principle of hydrodynamic focussing is given by Kachel and Menke, 1979, and Kachel et al., 1977.

A flow cell contains two flow streams. The outer stream called the carrier or sheath stream surrounds the sample stream. The sample stream is injected into the flow. The dual stream flow passes through a constriction. As long as the flow is laminar the inner stream

is constricted in the same relative amount as the carrier stream. Particles in the sample stream are thus confined to the central stream axis away from the walls. A particle free sheath fluid surrounds the inner nozzle accelerating and narrowing the inner stream. The degree of narrowing is given as follows. If A is the cross sectional area of the original sample stream, R is the radius of the outlet tube, and s is the distance from A to the tube outlet, then the area, a, of the sample stream in the outlet tube is given by

$$a = AR^2/2s^2$$

The principle of operation of the first type of sensor is described in Fig. 3. This cell type was developed by Sweet (Bonner et al., 1972). This cell used drop deflection methods based on ink jet technology. The cell suspension is injected through an inner nozzle with a diameter of the order of 50 to 100 μm. The coaxial streams emerge from the outer nozzle. Droplets are formed by vibrating the nozzle assembly at 40 kHz with a piezoelectric transducer. The 40 kHz frequency was chosen because the flow cell has a resonant frequency near this value. Uniform droplets are formed in synchronism with the excitation. For cell sorting, a charging collar is attached. The falling droplet stream is illuminated and sensed. If the droplet contained the particle of interest a high voltage (>1KV) deflector plate is activated deflecting the droplet path into a cell collector. This type of cell is favoured where flow sorting is required.

A second type of flow cell was developed based on quartz capillary tubes (Kamentsky, 1972; Shapiro et al., 1977). This arrangement is shown in Fig. 4. This type of cell is used principally for analysis. It consists of an inner and outer tube. The particles are sensed while still in the relatively long tube. The advantage of this scheme is that the particles are constrained to the central flow axis which permits more accurate focussing of the detection system onto the particle. However, the round capillary acts as a cylindrical lens

which reduces resolution unless a corrector is introduced. In order to circumvent this problem, variants of this flow cell type have been made using square or rectangular geometries most notably by the Los Alamos group (Gray et al., 1980). Commercial units such as the Ortho and the Coulter instruments use such a cell. Flow cells of this type are favoured for analysis.

Variations in the above two types have been made with circular, elliptical, square and rectangular flow nozzles. (Kay & Wheeless, 1977; Kachel et al., 1977). Some advantages accrue with one or more of the specific geometries if a particular cellular orientation is desired.

Several cells have been made for epi-illuminators. One such flow cell (Steen & Lindmo, 1979; Steen et al., 1980; Petersen, 1983) uses the same glass nozzle as used in the Sweet design (number 91216-x, Specialty Glass Products, Willow Grove, PA, USA) mounted obliquely with respect to a microscope slide. The water jet exiting the nozzle maintains its laminar flow past the microscope sensing volume. (See Fig. 5).

Another cell for a fluorescent microscope has the cell stream flowing upwards and then directed horizontally past the sensing volume. This was developed by Gohde (Steinkamp, 1984). A flow cell also to be used with a microscope using a glass capillary was developed by von Sengbush and Hugeman, 1974.

Another flow cell was developed in Germany for a microscope (Kachel et al., 1983). This cell is now available as part of a commercial fluorescence microscope based cytometry system (Leitz MPV).

A further flow cell was developed by another German group for the same purpose (Severin et al., 1983).

Several cells permit the use of Coulter counter conductivity techniques in conjunction with flow cytometry. (Fulwyler et al., 1969; Kachel et al., 1983).

6.0 ILLUMINATION AND BEAM FORMATION TECHNIQUES

Essentially all the earlier flow cytometer systems used laser illumination. Since the primary cell discrimination techniques are based on fluorescent dyes, the laser's high radiance and narrow spectral bandwidth are desirable characteristics. Additionally the laser beam may be focussed to a very fine spot. The most commonly used laser has been the gas ion type specifically either an argon ion or krypton ion laser. The principal wavelengths used are 488 nm and 514 nm for the argon laser and 647 nm, 531 nm and 415 nm for the krypton laser. These laser sources are the standard illumination sources used in the most common commercial instruments (e.g., BD FACS 400, Coulter Epiccs V). Older instruments such as the Biophysics instrument (no longer available) used helium-neon lasers to measure forward scattering. Helium-neon lasers while considerably cheaper than ion lasers produce less radiant power and the 633 nm output is distant from the absorption maxima of many of the most useful dyes. Dye lasers offer tunable narrow band illumination over the entire visible spectrum (Laser Dye Chart, Exciton Corp., Dayton, Ohio) with the use of multiple dyes. Unfortunately they require the use of a pump laser such as an ion laser for continuous operation. The expense is thus effectively doubled. Additionally laser dyes are relatively expensive and operating costs may be relatively high for high usage operations. Pulsed dye lasers are not useful for cytometry since the pulse rates of pulsed dye lasers is not fast enough in comparison to typical flow rates used in flow cytometry. For these reasons dye lasers have not been greatly used in cytometry.

Laser sources have permitted the detection of single dye molecules in flow cytometry experiments (Hirschfeld, 1976; Dovichi et al., 1983).

Multiple fluorescent dyes may be used if multiple wavelength sources which are spatially separated are used. (Dean, 1980). Separate wavelengths and beam spacings are required for multiple dyes or

quenching of the output of one dye's fluoresced radiation due to the other dye's absorption may result.

Typically, the laser is focussed onto the measurement zone using microscope objectives (Shapiro et al., 1977). Anamorphic lenses have been used in the multiple beam and in slit scan systems to provide elliptical beam cross sections with the short axis along the flow direction. (Cambier et al., 1979, Wheeless et al., 1980, Steinkamp et al, 1979).

This illumination is typically used in the multiple beam instruments. The short axis of the ellipse is along the flow direction. This allows the flow stream to wander and still be uniformly illuminated.

With microscope based systems using epi-illumination the epi microscope objective has been used. In some systems simultaneous epi and dia-illumination (straight through illumination) has been used (Kachel et al., 1982). Koper et al., 1982 used a zinc iodide modified Xenon arc lamp which produced high radiances at 468, 477.2 and 481 nm. They found that a Leitz H32/0.60 objective was particularly efficient at transmitting UV. Peters, 1979, did an intercomparison between a 100 W mercury arc illumination based cytometer and a 5 W argon-ion laser system. Peters concluded that Chromamycin A_3 and mithramycin were more efficiently excited by the laser and that Hoescht 33258 was about equally well suited to both the lamp and the laser. Additionally, the lamp had considerably lower capital and operational costs. The flow may be visualized using a flashlamp with a pulse duration of a few hundred nanoseconds or using a slit scan system (Kay et al., 1979; Cambier and Wheeless, 1979).

7.0 DETECTION METHODOLOGIES

The detection of cells using flow cytometry involves the collection of fluoresced or scattered radiation, the transduction and amplification of the radiation into an electrical signal, detection and

discrimination of the signal, storage of the information and, in the case of cell sorters, the activation of the sorting mechanism.

The collection of the radiation has been usually done by a microscope objective (Bonner et al., 1972) although simple cylindrical lenses (Sharpless et al., 1977) and ellipsoidal collectors have been used (Eisert, 1970; Skogen-Hagensen et al., 1977; Batholdi et al., 1980) in an effort to collect as much of the scattered or fluoresced radiation as possible. For the fluoresced radiation photomultiplier tubes are still preferred over silicon photodiodes due to the lower amplified noise. At low light levels, variation in the shot noise due to constant background light levels and not radiation collection efficiency is the limiting factor. For sources in the middle of the brightness range, photoelectron statistics control resolution and hence increased detector quantum efficiency, and increased optical numerical aperture would be of benefit. For high radiance sources, variations in the liquid stream control resolution. (McCutcheon and Miller, 1979). For scattered radiation, because higher light levels are available, photodiodes are preferred due to their relative stability and larger dynamic range.

Fluoresced radiation is usually believed (or at least assumed) to be isotropic so that radiation is collected in line with, perpendicular to, or in the reverse direction to the incoming beam. Transverse collection is usually preferred for maximum isolation between the source and fluoresced radiation. However, the experimental configuration may preclude such an arrangement as with fluorescent microscope based systems. With multiple detection wavelengths the radiation is usually collected by a common receiver optic and the incoming radiation is then split into several paths using beamsplitters (Kachel and Meier, 1980). The conventional arrangement is to use 45° angle beamsplitters in series. Such an arrangement is simple mechanically but 30° prism beamsplitters are superior due to polarization effects caused by beamsplitters mounted at 45° (Matteuci and Baumeister, 1980). This would be especially important if fluorescence polarization is used to establish cell structure (Eisert et al., 1980). Care in filter selection is

required since many glass filters are themselves fluorescent (Turner, 1973; Loken, 1980).

The relative angular distribution has been used to characterize cell structure. Backscattered radiation has been found to be more sensitive to cell structure (e.g., position of the nucleus) than scattering in the forward direction. (Kerker et al., 1979). It should be pointed out that the scattering coefficient is a generally increasing function of particle size but is not exactly monotonic (Bartholdi et al., 1980, Fig. 12, Cooke and Kerker, 1975). In practice the exact diameter is not desired but rather it is desired to know the particle diameter within a certain coefficient of variance. Thus peak height measurements of scattering do in fact give, within certain limits, a measure of particle size. This technique was more common in the earlier instruments (Mullaney et al., 1969).

Particle size is often measured using time of flight techniques which measure the leading and trailing edge of the particle. If the flow velocity is known, or calibration particles are introduced, the time of flight may be converted to a particle diameter. The electronics required in a typical time of flight instrument are shown in Fig. 6.

Typically, cells are measured in flow cytometry today with fluorescent tags. Kerker asked the question "Is the central dogma of flow cytometry true: that fluorescence intensity is proportional to cellular dye content" (Kerker et al., 1982). The answer is essentially yes. Thus peak height analysis of fluoresced radiation provides a measure of cell size.

Various schemes of pulse height analysis have been used, primarily those borrowed from nuclear spectroscopy, (e.g., constant fraction discriminator, Leary et al., 1979; Jett, 1980). Indeed many flow cytometers use multi-channel analyzers from the nuclear industry (e.g., the B-D Facs 400 uses instrumentation from Nuclear Data Inc.).

Once data is sensed, the information is invariably stored in computer memory.

In cell sorters, the decision making logic is most often accomplished using hardwired logic or EPROM (Erasable Programmable Read Only Memory) based logic due to the speed required (McCutcheon and Miller, 1982).

8.0 DATA ANALYSIS METHODOLOGIES

Once data has been collected, the data analysis revolves around the most effective method of multivariate data analysis. Typically this involves a combination of statistical and graphical interpretation. Typically the fluorescent intensity data are plotted against time of flight or size data; the fluorescent intensity are plotted against the scattered radiation or against the fluorescent radiation at another wavelength band. Box plots (Jett et al., 1980), histograms (Bryant et al., 1980) or 3-D plots may be generated (Valet, 1980). With commercial systems one may select regions in a graph and count densities may be computed. In some analysis and modelling packages, linear and nonlinear least squares analysis may be performed to define a model (e.g., Paral and Modset instructions in Coulter Electronics "Easy88" package). Multiple analysis of variance may also be performed. Murphy and Chused, 1983, proposed a data file standard to facilitate data comparisons.

9.0 DESCRIPTION OF COMMERCIAL "TURNKEY" SYSTEMS

There are essentially three main manufacturers of flow cytometric equipment:

1. B-D Facs
   490-B Lakeside Drive
   Sunnyvale, CA 94086
   USA

2. Ortho Diagnostic Systems Inc.
   410 University Ave.
   Westwood, MA 92090
   USA
3. Coulter Electronics Inc.
   P.O. Box 2145
   Hialeah, FL 33012-0145
   USA

   in Canada Coulter Electronics
   905 Century Dr.
   Burlington, Ont.
   L7L 5JB

B-D Facs make instruments originally based on the Stanford design (Bonner et al., 1972). The first group of instruments made by B-D Facs are called the 400 series which include three instruments - the 400, the 420 and the 440. All three instruments offer essentially the same "front end" components. The major differences are in the computer hardware and software with the 440 instrument being the most advanced. Additionally B-D Facs make what they call the FACS Analyser which is primarily used for whole blood analysis and does not offer cell sorting capapibilities. The cheapest B-D system is well over $100 K Canadian. B-D Facs is generally regarded to have the best flow cell for sorting purposes.

Ortho make three flow cytometers. The first two are the CYTOFLUOROGRAPH II and the CYTOFLUOROGRAPH IIs which appear to be newer versions of the older Biophysics Inc. Cytofluorograph originally developed by Kamentsky and Adams, 1972. Multiple lasers may be used and both forward and 90° measurements may be made. The third instrument is called the ORTHO SPECTRUM III which is a somewhat simplified version of the CYTOFLUOROGRAPH. Data acquisition and data analysis are done using their 2140 or 2151 Data Handlers, with the 2151 being the most comprehensive. The Ortho flow cell appears to be a modification of the Lawrence Livermore design (Gray et al., 1980). This cell contains quartz windows. The flow cell is generally regarded as being the best flow cell for analysis of the "big three" manufacturers but the worst for cell sorting. The Ortho software is regarded as being the best of

the three main manufacturers. Costs typically are of the order of $150 K to more than $300 K Canadian depending on the version purchased.

Coulter make two instruments. The first instrument is called the EPICS C which is oriented toward clinical use. Sorting protocols may be changed using software. Forward and 90° scatter as well as two 90° fluorescence channels may be used. The EPICS V is similar except that a more advanced data acquisition and analysis system, which Coulter calls MDADS, is used. Coulter is regarded in having a reasonably good cell for sorting and reasonably good data analysis. Costs of the instruments vary between $150 K to $250 K Canadian or even more depending on the options desired.

There were several other manufacturers at one time including Biophysics Inc. and Phywe AG both of which were purchased by Ortho. Additionally Technicon Inc. make a flow cytometer specifically for blood analysis called the Hemalog D (Technicon Inc. Tarrytown, NY, USA). Leitz also make a flow cytometer adapter for one of their fluorescent microscopes. Their system is called "Leitz-MPV" and appears to be based on the Max Planck Institute flow cell (Kachel et al., 1982). The Canadian contact, Wild Leitz, 513 McNicoll, Toronto, Ontario, was approached for information but none was provided.

10.0 UTILITY OF THE METHOD FOR PHYTOPLANKTON AND BACTERIAL MEASUREMENT

Of the many hundreds of papers in flow cytometry only a very small number have been devoted to the measurement of phytoplankton and bacteria. This is so in spite of the fact that phytoplankton is a very good candidate for flow cytometry since it has a built-in highly fluorescent material, chlorophyll-a, and contains additional photopigments such as phaeophytin and carotenes.

However, a number of researchers have used the technique with apparent success. Paau, Oro, and Cowles, 1978, used the difference in chlorophyll fluorescence to obtain size distribution of Quadruplicatum,

Trebouxia, Chlorella pyrenoidose and Euglena gracilis. A peak height discrimination system based on a system design by Steinkamp et al., 1973, for mammalian cells was used. Additionally the light scattering technique was also used and differences in the relative scattering intensity between species was noted. Ethidium bromide fluorescence was used as a measure of nucleic acid content. However, de-chlorophyllization was required however to circumvent the non-additive fluorescence of the chlorophyll-a. De-chlorophyllization was done using acetone. Ozone could also have been used (Pak et al., 1981). Fluorescein isothiocyanate was used as an indicator of protein content. Paau et al., 1979, used ethidium bromide for bacteria measurements. Rhizodium japonicum and Bacillum subtilis were used.

Olson et al., 1983, used a system based on Shapiro's system (Shapiro et al., 1977) and Arndt-Jovin and Jovin's technique (Arndt-Jovin and Jovin, 1977) to measure the DNA content of a marine diatom, a coccolithophore, and a dinoflagellate. As with the ethidium bromide, the chlorophyll absortion quenched the Hoescht 33342 dye fluorescence giving anomalous results unless the cells were de-chlorophyllized first. Propidium iodide, which cannot enter viable cells but does label the DNA content of dead cells (Loken et al., 1980) was also used by Olson et al., but with no improvement over the Hoescht 33342 dye.

Hutter and Eippel, 1978, used an Ortho "CYTOFLUOROGRAPH" and propidium iodide, fluorescein isothiocyanate, erythrosine B and chlorophyll content to measure intracellular substances. They noted that care is required in the method to ensure that only single cell populations are examined and that pre-treatment is required for cells which grow in chains, pairs, clusters or in filamentous forms and clumps.

Van Dilla et al., 1983, used a double staining of chromomycin A_3 and Hoescht 33258 to determine QC/AT ratios. Chromomycin A_3 preferentially binds to quanine-cytosine (QC) and Hoescht 33258 binds to adenine-thymine (AT). Six bacterial cultures were

examined and the authors claim they may be distinguished on this basis. Boye et al., 1983 used mithramycin/ethidium bromide as a measure of DNA content in E. coli and determined that scattered light was proportional to cellular protein content.

Live cell/dead cell measurement is important in cell population determinations. Fluorescein diacetate, trypan blue or Hoescht 33662 in combination with propidium iodide have been used in mammalian cells for this purpose (Stohr and Vogt-Schaden, 1980). Nigrosin dye has also been used.

It is clear that a large measure of the success or failure of flow cytometry to distinguish between cell populations is due to the success or failure of dye staining methodologies.

11.0 DESCRIPTION OF A PROPOSED "HOME-BUILT" SYSTEM

The first flow cytometers (e.g., Bonner et al., 1972; Kamentsky, 1972) were of necessity home built systems. Throughout the evolution of flow cytometry there have been a number of special purpose and "home built" cytometers built either because of cost considerations or with a view to advancing the state-of-the art (e.g., Miller et al., 1981).

With the advent of commercial instruments the rationale for developing an instrument remains the same, i.e., cost and state-of-the-art advancements. However the commercial instruments provide a clear benchmark against which to compare any custom-built system. While some environmental research establishments can afford commercial gear (Bedford Institute of Oceanography have procured a Coulter instrument for phytoplankton measurement in the laboratory and on board ship) it is clear that the majority of marine and limno biologists cannot afford the $150 K to $300 K needed to procure such a system. In many institutions, it would require that a research or environmental impact program would have to revolve around the instrument rather than the instrument revolve around the program, assuming that the

instrument could be afforded in the first place (R. Daley, personal communication). While much work has been done in applying flow cytometry to medical research as the previous section pointed out, little work has been done in phytoplankton measurement using this technique. Thus even if a "turn key" commercial system were purchased, a risk remains that a relatively large expenditure might be wasted. Moreover, no commercial instrument has been optimized for phytoplankton or bacteria measurement. No protocols such as those available from Ortho Diagnostics for lymphocytes, leuocytes, phagocytosis or immunologlobulins exist for plankton or bacteria. Thus even with commercial systems, development work to establish the most reliable methods would still have to be done. It would appear that if flow cytometry is to have an impact on traditional methods of cell counting in marine biology, a lower cost instrument is required.

A flow cytometer may be broken down into four parts: 1) the flow cell, 2) the illumination and detection optics, 3) the detection and data acquisition electronics, and 4) the computer and analysis routines.

In most commercial instruments, cell sorting is offered. This puts constraints on the optics and requires much effort on the data acquisition and sorting algorithms. If cell sorting is not required, the system may be simplified, and much off-the-shelf optics may be used. A number of systems based on a fluorescent microscope were discused in Section 6. Such a system was constructed for phytoplankton work (Olson et al., 1983). The adaptation of a fluorescent microscope as the basis of the optical train of a flow cytometer appears to be a useful idea because: 1) excellent optics are available at a moderate price, 2) most marine biology laboratories already have one or more fluorescent microscopes. Additionally, experienced flow cytometer users recommend that it is good practice to keep a microscope close to any flow cytometer to verify that cell aggregates have not formed (Miller et al., 1981).

Today, desktop computers and data acquisition electronics with memory (sufficient to count several hundred thousand cells) are available for less than $5 K Canadian. A plug-in data acquisition board which will digitize 16 channels of analog data with 12 bit resolution with direct memory access is available for less than $2 K Canadian including driving software (e.g., Quatech Inc., Akron, Ohio). Much statistical software such as SPSS (Statistical Package for Social Sciences) or IMSL is available for less than $1.5 K for these machines. These packages offer cluster analysis routines and linear and non-linear regression analyses (e.g., Levenberg-Marquardt analysis) which are used with commercial flow cytometers. It is evident, therefore, that a substantial amount of a flow cytometer system could be assembled from either existing gear such as the microscope or from relatively low cost components.

However, the flow cell and the front end electronics cannot be purchased cheaply.

The front end electronics are available but are expensive. However, time of flight and/or peak height electronics are relatively easy to design for flow cytometry. This is because of the relatively slower rates required in comparison to nuclear spectroscopy of photon counting electronics. Such electronics does more than what is required of flow cytometer "front-end" electronics and has a higher price.

Numerous cytometric flow cells have been described in the literature and were discussed in Section 6. The easiest cells to construct are that of Steen and Lindmo, 1979, and that designed by Shapiro and used by Olson et al., 1983, in their phytoplankton measurement system. It would seem desirable to replicate these two cells initially. The disadvantage of the Steen and Lindmo cell is that there is no provision for waste collection. The disadvantage of the Shapiro cell is that it is good for large particles but cannot be used for bacteria.

Thus some modifications to these designs would likely be required. Because it is unlikely that a single flow cell could accommodate the 1-100 μm dynamic range, multiple cells would be required.

The associated plumbing required has been described to some extent by Dean (Dean, 1985) and by Miller (Miller et al., 1981). The sheath flow could be provided using tap water filtered through 0.22 μm filter cartridges (e.g., Barnstead "Nanopure" filters available from Cole-Parmer, Chicago) and pressure regulated by reduction valves and accumulators. The sample would be in a glass vessel and stirred using a Markson scientific stirrer for instance. The sample would be injected using a stepping motor syringe such as model 352 from Sage Instruments, White Plains, NY. The flow would be measured using a flow meter available from a company such as Gilmont.

A Leitz "Ortholux" microscope could be used. This is a commonly available microscope available in many government and university laboratories in Canada. The actual microscope would depend on availability. Modifications required would include the modification of the photographic head (tri-ocular tube) to incorporate an optic containing a beamsplitter assembly, spectral filters, PMT's such as Hammamatsu R647's and a photodiode such as an EG&G UV-100. Support bracketry would stiffen the structure. A mercury arc source and power supply would be purchased (e.g., from PRA, 45 Meg Dr., London, Ontario) and modified to include photoelectric stabilization of the arc irradiance. Such a feature is readily implemented using a photodiode and amplifier as the reference element in the arc lamp regulator circuitry. Such a feature is desirable since the detection threshold in high signal situations is dependent on flow and photometric stability, as discussed in Section 8.

12.0 CONCLUSIONS

Several methods of examining cell "biomass" were examined, including traditional microscopy, pattern recognition, and flow cytometry. Flow cytometry appeared to be an attractive supplement to traditional microscopy. The literature and commercial systems were reviewed. Commercial systems at this writing were believed to be too expensive for most laboratories. A "home built" system is proposed and some of the system components are enumerated.

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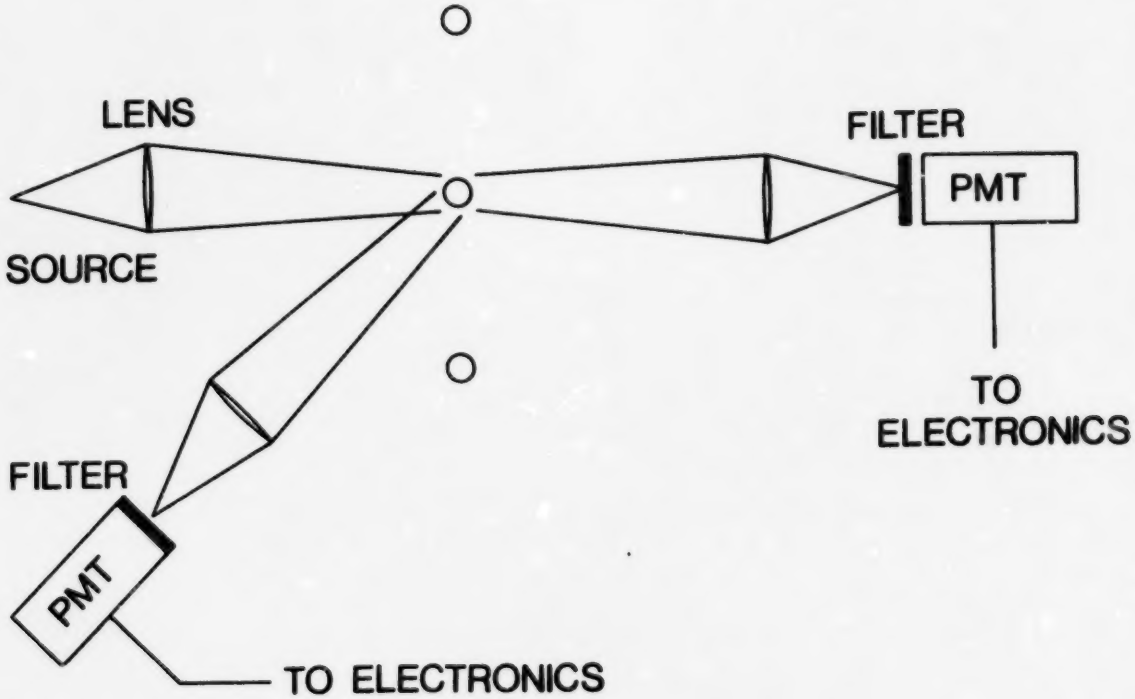
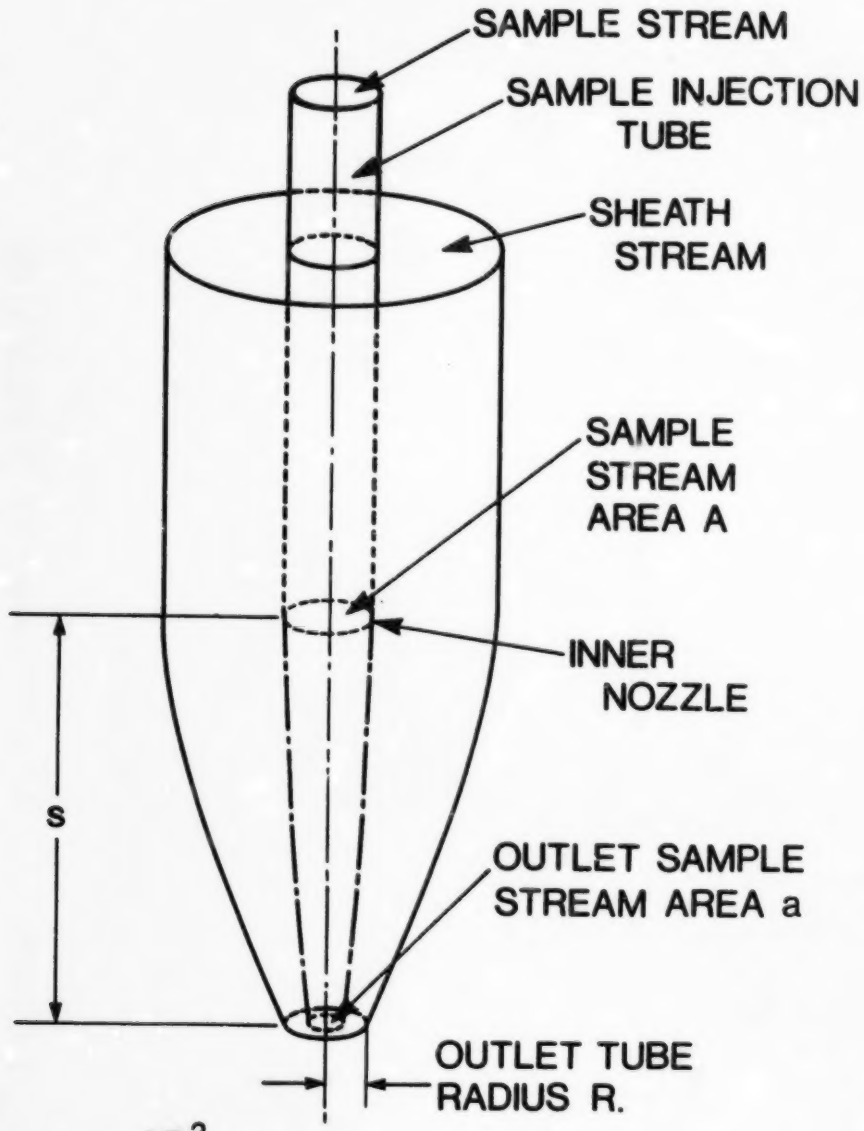


FIGURE 1. FLOW CYTOMETRY SCHEMATIC



$$a = \frac{AR^2}{2s^2}$$

FIGURE 2. HYDRODYNAMIC FOCUSING

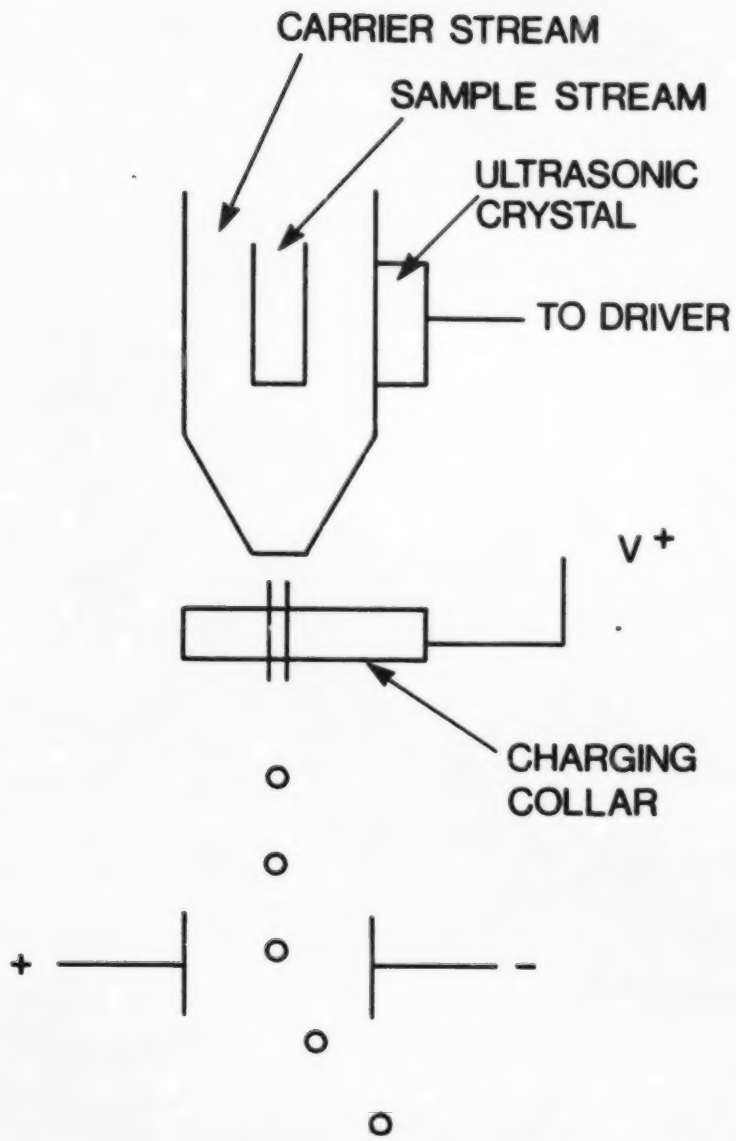


FIGURE 3. SWEET CELL

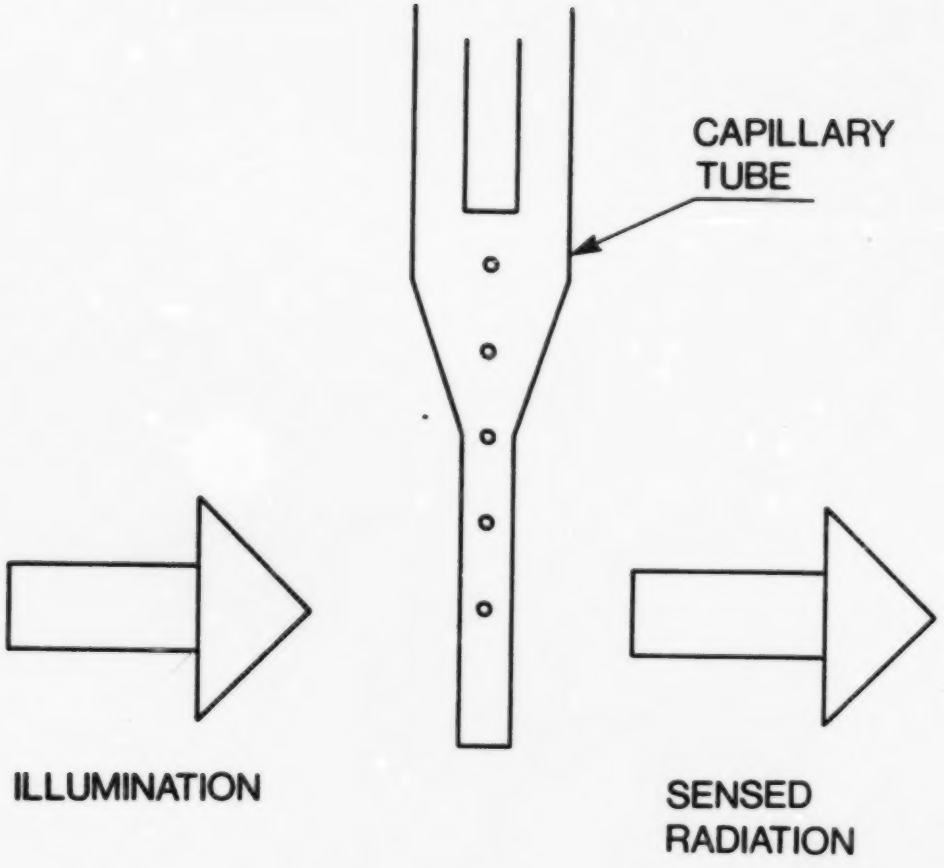


FIGURE 4. CAPILLARY TUBE FLOW CELL

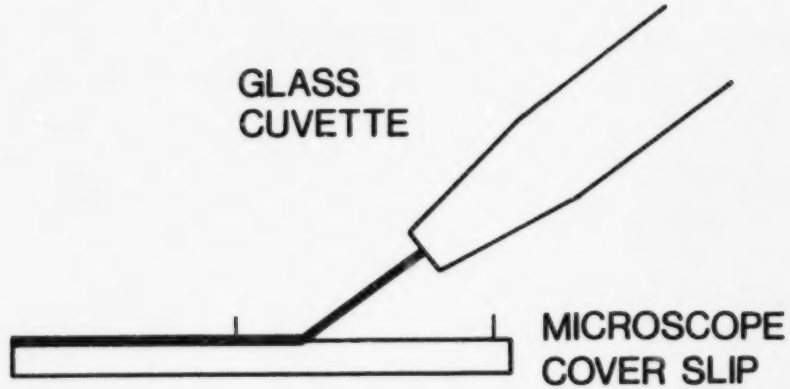


FIGURE 5. STEEN AND LINDMO CELL

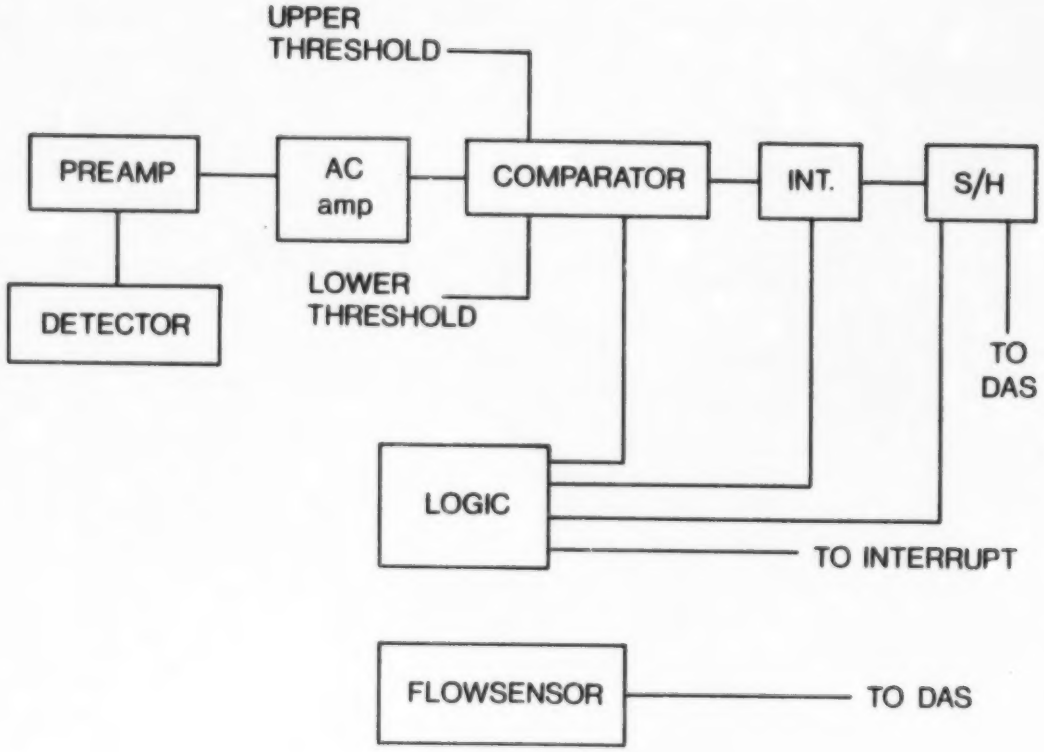


FIGURE 6. TIME OF FLIGHT DETECTOR BLOCK DIAGRAM